REMARKS

In view of the above amendments and the following remarks, reconsideration of the outstanding office action is respectfully requested.

More than 2,000 conditions have been identified as single-gene defects for which the risk of producing affected offspring can be mathematically predicted. Among these conditions in man include Huntington's chorea, cystic fibrosis, $alpha_1$ antitrypsin deficiency, muscular dystrophy, Hunter's syndrome, Lesch-Nyhan syndrome, Down's syndrome, Tay-Sachs disease, hemophilias, phenylketonuria, thalassemias, and sickle-cell anemia.

Three important techniques have been developed recently for directly detecting these single nucleic acid base pair changes, deletions, insertions, translocations or other mutations. However, two of these techniques cannot be easily automated. In the first such technique, the presence or absence of the mutation in a patient's clinical sample is detected by analysis of a restriction digest of the patient's DNA using Southern blotting. However, the Southern blotting technique cannot be used for genetic diseases where the mutation does not alter a restriction site as, for example in $alpha_1$ antitrypsin deficiency. The second technique is by the use of DNA probes which involves the synthesis of an oligonucleotide of about 19 base pairs that is complementary to the normal DNA sequence around the mutation site. The probe is labelled and used to distinguish normal from mutant genes by raising the stringency of hybridization to a level at which the probe will hybridize stably to the normal gene, but not to the mutant gene, with which it has a single base pair mismatch. The original method has been modified by immobilizing the oligonucleotide and probing with a labelled PCR amplified sample. In this modification, the sample is allowed to hybridize to an immobilized oligonucleotide and is then washed off by raising the stringency of hybridization as described above. Other methods have been developed which use fluorescent PCR primers to specifically amplify only one mutation or allele. This method requires the separation of products from primers by spin columns or gel electrophoresis and, hence, is not amenable to large scale automation. The third technique utilizes the presence of both diagnostic and contiguous probes under conditions wherein the diagnostic probe remains substantially covalently bound to the contiguous probe only in the case wherein the sample nucleic acid contains the exact target sequence. In addition, the diagnostic oligonucleotide probe may contain a "hook" (for example, a biotinylated oligonucleotide) which is captured (for example, by streptavidin) as a means of increasing the efficiency of the technique, and the contiguous probe may contain a detectable moiety or label.

Although it is not always necessary, the detection of single base pair mutations in DNA is usually preceded by techniques to increase or amplify the amount of DNA sample material. A number of techniques exist to perform nucleic acid amplification, among which are: (1) polymerase chain reaction, which can amplify DNA a million fold from a single copy in a matter of hours using *Taq* polymerase and running 20 to 30 reaction cycles on a temperature cycling instrument; (2) self-sustained sequence replication or 3SR, which can amplify DNA or RNA 10 million fold from a single copy in less than an hour using reverse transcriptase, T7 RNA polymerase, and RNase H under isothermal conditions at 37°C.; and (3) Q Beta Replicase, which can replicate a few thousand RNA molecules containing a special 300bp recognition sequence a billion fold in 30 minutes. Additional techniques are available, and one, the ligase chain reaction, is discussed in the present application, which describes the cloned thermophilic ligase according to the present invention.

In addition to various genetic diseases that may be diagnosed utilizing the present invention, various infectious diseases can be diagnosed by the presence in a clinical sample of a specific DNA sequence characteristic of the causative microorganism. These include bacteria, viruses, and parasites. In such procedures, a relatively small number of pathogenic organisms may be present in a clinical sample from an infected patient and the DNA extracted from these organisms may constitute only a very small fraction of the total DNA in the sample. However, specific amplification of suspected pathogen-specific sequences prior to immobilization and detection by hybridization of the DNA samples should greatly improve the sensitivity and specificity of traditional procedures. In addition, amplification is particularly useful if such an analysis is to be done on a small sample using nonradioactive detection techniques which may be inherently insensitive, or where radioactive techniques are employed, but where rapid detection is desirable.

Although techniques such as these are available, the search for other techniques for determining single base pair mutations continues. The present invention, that involves DNA amplification and/or detection by a ligase detection reaction (LDR) or ligase chain reaction (LCR) utilizing the thermophilic DNA ligase from *Thermus aquaticus* to detect a target DNA sequence, is part of that continuing effort.

Although other techniques utilizing *E. coli* or T4 DNA ligase for DNA amplification have been attempted, these have been found to be unacceptable because of a high background "noise" levels (after as few as 10 cycles), a condition which does not exist in the ligase chain reaction according to the present invention.

DNA amplification and/or detection has also been attempted utilizing specific ligases. For example, a ligase amplification reaction has been reported that can amplify DNA

starting with 500,000 copies in 95 hours, using 75 cycles and replenishing the T4 DNA ligase used after each cycle. However, this reported technique is slow and requires the addition of fresh T4 ligase at each step, both of which requirements make this reported technique unacceptable for automation. The ligase chain reaction according to the present invention allows for amplification of DNA from 200 copies in 3 hours using 30 cycles and does not require the addition of ligase following each cycle.

The present invention is intended to overcome the deficiencies of prior art genetic defect detection procedures.

Applicants submit that the above amendment to the specification updates the status of the parent application as requested by the U.S. Patent and Trademark Office ("USPTO").

The rejection of claims 5 and 6 under 35 U.S.C. § 112 (2nd para.) for indefiniteness is respectfully traversed.

It is the position of the USPTO that the recitation of the phrase "suitable for ligation" is unclear. Applicants disagree.

One of ordinary skill in the art, having read the present application, would have understood what "suitable for ligation" means in the context of claim 5. The specification teaches that the two oligonucleotide probes in a given "probe set" are allowed to hybridize to a denatured DNA target strand such that the 3' end of one probe is immediately adjacent to the 5' end of the other probe following hybridization (pg. 40, lines 18-21). When ligase is provided, a covalent phosphodiester bond is formed between the two oligonucleotides "provided that the nucleotides at the junction are perfectly complementary to the target" (pg. 40, lines 23-26). On the other hand, if the oligonucleotide probes hybridize to a nucleic acid molecule with a mismatch at their junction, no ligation will occur (pg. 40, lines 28-30). Thus, a skilled scientist, having read the present application, would have understood that the "at least two oligonucleotides suitable for ligation together at a first ligation junction" limitation in claim 5 are oligonucleotides that can ligate together when they hybridize in a juxtaposed position to a target nucleotide with perfect complementarity to the target at their junction.

Since the meaning "suitable for ligation" is capable of being fully understood by one of ordinary skill in the art, the indefiniteness rejection based on that phrase is improper and should be withdrawn.

It is also the position of the USPTO that of the phrases "denaturation treatment," "thermal hybridization treatment," and "detecting the presence" of a nucleic acid

are not clear without reference to conditions under which the steps are practiced. Applicants disagree.

Applicants submit that one ordinary skill in the art, having read the present application, would have understood fully how to carry out the claimed invention. Methods for carrying out a "denaturation treatment" or a "thermal hybridization treatment," on a nucleic acid molecule, as well as DNA detection, are disclosed in extensive detail in the present application and were well known at the time the present invention was made.

Firstly, denaturation and hybridization treatments of target (double-stranded) DNA are described in the present application at pg. 5, lines 24-30 as follows:

> at very high temperatures such as 94°C, virtually all double stranded DNA (independent of length) unwinds and melts. If one cools the temperature (to 45-65°C) in the presence of complementary oligonucleotides, they can hybridize to the correct sequences of the unwound melted DNA. DNA that has been melted and cooled in the presence of complementary oligonucleotides is now a substrate for the DNA ligase reactions.

As taught in the present application at pg. 14, lines 6-11, and well-known to those skilled in the art, the exact heating conditions needed for denaturation depend upon a number of sample-dependent factors, including the length and the nucleotide composition of the nucleic acid being denatured. As a skilled scientist would have known, the exact temperature at which two complementary strands of DNA unwind and separate during the denaturation process is referred to as the "Tm" of the DNA. The Tm for a given double-stranded DNA template can be determined as taught in the specification at pg. 13, lines 3-7. The specification also teaches conditions for denaturation and hybridization in Example XI.

For all the above reasons, applicants submit that one skilled in the art would have understood what is meant by the terms "denaturation treatment" and "thermal hybridization treatment" as recited in claim 5 of the present invention.

Applicants submit that those skilled in the art, having read the present application, would have readily understood what is meant by the phrase "detecting the presence of ligated oligonucleotides of the first oligonucleotide set." A multitude of methods for detecting the presence of nucleic acids were well known at the time the present invention was made. For example, as taught by the present application, gel electrophoresis using radiolabeled samples (*see* Examples IX and X, and Figures 3-5) and various non-radioactive detection methods that result in colorimetric, chemiluminescent, or fluorescent products (*see* pg. 43, lines 15-20; pg. 61, line 14 to pg. 68, line 5; and Figure 6) are suitable detection techniques. Other methods not disclosed in the specification would have been well within the abilities of a skilled scientist at the time the present invention was made.

Since one skilled in the art would have understood what was meant by "a denaturation treatment," "a thermal hybridization treatment," and "detecting the presence" of an oligonucleotide, it is submitted that the rejection under 35 U.S.C. §112 (2nd para.) based on these limitations is improper and should be withdrawn.

The rejection of claims 5-6 under 35 U.S.C. § 112 (1st para.) for lack of enablement is respectfully traversed.

Applicants submit that the making of the claimed invention is taught in such complete and sufficient detail in the present application that one of ordinary skill in the art would have been fully able to make and use the present invention.

As amended, claim 5 is drawn to "[a] method for detecting a first nucleotide sequence which differs from a second nucleotide sequence" that includes "providing a thermocyclable ligase which does not become irreversibly denatured and lose its catalytic activity when subjected to temperatures ranging from about 50° C. to 105° C.; blending the sample, the first oligonucleotide set, and the ligase to form an amplification mixture; subjecting the amplification mixture to a series of cycles comprising a denaturation treatment, and a thermal hybridization treatment; and detecting the presence of the first nucleotide sequence in the sample by detecting the presence of ligated oligonucleotides of the first oligonucleotide set." Thus, the claimed invention is not directed to a new ligase but to a method of detection.

The present application discloses, in extensive detail, the preparation of a thermocyclable ligase that "does not become irreversibly denatured and lose its catalytic activity when subjected to temperatures ranging from about 50° C. to 105° C." It describes the isolation of genomic DNA from an exemplary thermophilic bacterium (Example I); the preparation of multiple vector libraries containing the DNA and selection and analysis of clones (Examples II-IV); the isolation, purification, and sequencing of the cloned thermocyclable ligase nucleic acid sequence and amino acid sequence, and subcloning of the nucleic acid molecule into an overexpression vector (Example V), and purification of the thermocyclable enzyme (Example VI). The specification also discloses methods for assaying the specificity of a thermocyclable ligase (Examples VII-IX) and the use of a thermocyclable enzyme "for detecting a first nucleotide sequence which differs from a second nucleotide sequence" (Examples X-XI), including denaturation and hybridization conditions for a thermocyclable enzyme (*see* description above and Example XI). Applicants submit that it would have been well within the capabilities of one skilled in the art to determine, without undue experimentation, whether a given ligase is thermocyclable, i.e., whether it does or does

not become irreversibly denatured and lose its catalytic activity when subjected to temperatures ranging from about 50° C. to 105° C.

In addition, the application discloses in Example V that it is known in the art that thermophilic proteins may be substantially modified and still retain sufficient activity for use in the present invention (pg. 34, line 29 to pg. 35, line 12). Thus, one skilled in the art would expect that following the guidance provided in the specification, thermocyclable ligases other than those having SEQ ID NO: 2 and NO: 8 could be prepared and used in the claimed invention. In particular, a skilled scientist would have known the specific characteristics to look for in such a ligase, because the specification teaches that a ligase in accordance with the present invention is useful for amplifying DNA and discriminating single base substitutions in a DNA (*see* page 40, lines 7-10 of the present application). It further teaches that "[t]he single most important property" of the ligase of the present invention is that it "retains activity during repeated thermal denaturation/renaturation cycles thus allowing for the amplification of DNA without necessitating repeated addition of ligase" (*see* page 40, lines 10-13 of the present application). Moreover, the ligase of the present invention will ligate oligonucleotides of a length which is sufficient to assure their uniqueness in complex genomes at or near the T_m temperatures of 65°C, and will also accurately discriminate between exactly complementary and single based mismatched oligonucleotide sequences." (*See* pg. 40, lines 10-17.)

From the foregoing, it is apparent that the present application contains extensive ligase disclosure — largely to support claims (not pending) which are directed to a ligase *per se*. However, with regard to the claimed method of detecting, it is clear that applicants did not try to patent a new ligase, but, rather, a new and unobvious method for detection useful with a variety of thermocyclable ligases. Having made a discovery of this broad nature, it is entirely inappropriate to attempt to limit patent coverage for that method to specific ligases when others would clearly work.

For all these reasons, the rejection under 35 U.S.C. § 112 (1st para.) for lack of enablement is improper and should be withdrawn.

The rejection of claims 5-6 under 35 U.S.C. § 112 (1st para.) for failure to meet the written description requirement is respectfully traversed.

It is the position of the USPTO that applicants have not described the ligase recited in claims 5-6 in sufficient detail to show that applicants were in possession of the invention as claimed. Applicants disagree.

Applicants submit that one of ordinary skill in the art, having read the present application, would have understood that the inventors were in full possession of the invention

as claimed. The rejection for failure to satisfy the written description requirement is yet another inappropriate attempt to limit the claims to the ligases actually utilized when it is readily apparent the other thermocyclable ligases could also be used. What applicants made and claim here is a new and unobvious method - - not a ligase. Therefore, the claims should not be limited to the ligases exemplified by the present application.

As described above, the present application teaches, in detail, how the claimed ligase is made and used. One of ordinary skill in the art, having read the present application, would understand that at the time the application was filed, applicants were in possession of the claimed method. Therefore, the rejection of claims 5-6 under 35 U.S.C. § 112 (1^{st} para.) for failure to meet the written description requirement should be withdrawn.

The rejection of claim 5 under 35 U.S.C. § 102(b) as anticipated by Landegren et al., "A Ligase-Mediated Gene Detection Technique," *Science* 241:1077-80 (1988) ("Landegren") or Wu et al., "Specificity of the Nick-Closing Activity of Bacteriophage T4 DNA Ligase," *Gene* 76:245-254 (1989) ("Wu"), is respectfully traversed in view of the above amendments and the following remarks.

Landegren discloses an assay for detecting the presence or absence of a mutant nucleic acid in a test sample using oligonucleotide probes selected to anneal to immediately adjacent segments of a target nucleic acid. Ligase is added so that if the oligonucleotides are correctly paired at their junction, they are covalently joined. If pairing at the junction is incorrect, the probes are not joined. The presence or absence of a defect is detected. The process of Landegren does not involve cycles comprising a denaturation treatment and a thermal hybridization treatment.

Wu discloses a ligation amplification reaction process in which oligonucleotide pairs that are complementary to adjacent cites on an appropriate DNA template are hybridized to the template and ligated to one another at a temperature of around 15°C with T4 ligase. The hybridized sequences are then dissociated by denaturation at a temperature of around 100°C, during which the T4 ligase is destroyed, and fresh enzyme must be added prior to each new hybridization stage in order for the process to proceed.

Neither Landegren nor Wu teach or suggest a method of detecting using a thermocyclable ligase which does not become irreversibly denatured and lose its catalytic activity when subjected to temperatures ranging from about 50° C. to 105° C. Since Landegren and Wu do not anticipate the claimed invention, the rejection under 35 U.S.C. § 102(b) is improper and should be withdrawn.

The rejection of claim 6 (the limitations of which are now recited in amended claim 5) under 35 U.S.C. § 103(a) for obviousness over Landegren or Wu in view of

Takahashi et al., "Thermophilic DNA Ligase Purification and Properties of the Enzyme from Thermus Thermophilus HB8," *J. Biol. Chem.* 259:10041-10047 (1984) ("Takahashi") is respectfully traversed.

Takahashi discloses a thermophilic DNA ligase purified from the extract of *Thermus thermophilus* HB8. It is demonstrated that this ligase can catalyze the formation of phosphodiester linkages between DNA chains. The ability of this ligase to join thymidylate oligomers in the presence poly(dA) as a template is demonstrated with the nick-closing activity of this enzyme having a temperature range of 15-85°C, optimally 65-72°C.

A proper *prima facie* showing of obviousness requires the USPTO to satisfy three requirements. First, the prior art relied upon, coupled with knowledge generally available to one of ordinary skill in the art, must contain some suggestion which would have motivated the skilled artisan to combine or modify references. *See In re Fine*, 837 F.2d 1071, 1074, 5 USPQ2d 1596, 1598 (Fed. Cir. 1988). Second, the USPTO must show that, at the time the invention was made, the proposed modification had a reasonable expectation of success. *See Amgen v. Chugai Pharm. Co.,* 927 F.2d 1200, 1209, 18 USPQ2d 1016, 1023 (Fed. Cir. 1991). Finally, the combination of references must teach or suggest each and every limitation of the claimed invention. *See In re Wilson*, 424 F.2d 1382, 1385, 165 USPQ 494, 496 (CCPA 1970).

There is no suggestion in Takahashi that its ligase is useful in carrying out the procedure disclosed by Landegren and Wu. Takahashi is simply directed to a process of ligating oligomers in the presence of a complementary template. There is no suggestion in Takahashi that its ligase has utility in Landegren's detection technique or in a ligation amplification reaction process like that of Wu. More particularly, there is no indication in Takahashi that its HB8 DNA ligase would not interfere with, let alone be useful in, steps of hybridizing oligomers to a DNA template or denaturing strands of hybridized DNA.

The rejection of the claims based on the combination of Landegren, Wu, and Takahashi constitutes nothing more than an impermissible "obvious to try" position. *See In re Yates*, 663 F.2d 1054, 211 USPQ 1149 (CCPA 1981). As noted in *In re O'Farrell*, 853 F.2d 894, 7 USPQ2d 1673 (Fed. Cir. 1988), a rejection by the USPTO is erroneous when:

> what was "obvious to try" was to explore a new technology or general approach that seemed to be a promising field of experimentation, where the prior art gave only general guidance as to the particular form of the claimed invention or how to achieve it.

Id. at 903, 7 USPQ2d at 1681. This is precisely the situation here. As noted above, neither Landegren nor Wu teach or suggest the use of a thermocyclable ligase in its gene detection technique. Even if one skilled in the art would have thought to combine the thermostable ligase of Takahashi in Landegren's detection technique or in a ligation amplification reaction process like that of Wu, which they would not have, even in combination the references provides absolutely no guidance on how to identify a ligase useful in the methods taught by Landegren and Wu. Instead, the reader is left to choose amongst available enzymes and try to find one which is useful. This is hardly sufficient guidance to enable one of ordinary skill in the art to proceed. The disclosure of Takahashi is of no help, because, as noted above, there is no indication that the HB8 ligase disclosed in that reference would not interfere with, let alone be useful with, the hybridization and denaturation steps of Wu's ligation amplification reaction. Thus, the obviousness rejection based on Landegren or Wu and Takahashi follows an impermissible "obvious to try" approach and should be withdrawn.

With regard to claim 5, as amended above, the attached Declaration of Francis Barany under 37 CFR § 1.132 ("Barany Declaration") demonstrates the benefit of having the distinguishing nucleotide be complementary to the oligonucleotide of the first oligonucleotide set having its 3' end at the first ligation junction, in accordance with the claimed invention.

The Barany Declaration describes a fluorescent assay for quantitative analysis of the fidelity of *Thermus thermophilus* ("*Tth*") DNA ligase against every possible mismatch on both the 3' and 5' side of a nick (Barany Declaration ¶ 4). The analysis is described in the Barany Declaration (¶¶ 5-10). The fidelity results on the 3' side of the nick demonstrate that the yield of all mismatches was less than 15% even after 23 hours of incubation, while the yield was near 90% for wild type enzyme within two hours (Barany Declaration ¶ 11). Only T-G and G-T mismatches accumulated at a yield of about 15% over a 23 hour incubation period, while the remaining mismatches were less than 5% (*Id.*). In other experiments (not shown) with an A-T match, yields of product using wild type enzyme were 79% after 15 minutes, 85% after 30 minutes, 88% after 1 hour, and 92% after 2 hours (*Id.*). With a C-G match, such yields were 66% after 15 minutes, 77% after 30 minutes, 81% after 1 hour, and 90% after 2 hours (*Id.*). When mismatches were located on the 5' side of the nick, the fidelity pattern of the *Tth* DNA ligase was quite different, with many mismatches increasing dramatically (Barany Declaration ¶ 12). Thus, *Thermus thermophilus* DNA ligase discriminates mismatches at the 3' side of the nick much more efficiently than those placed on the 5' side of the nick (*Id.*). This is true even with respect to the less efficiently discriminated against T-G and G-T mismatches (*Id.*). It would have been expected that fidelity would have been better for mismatches at the 5' side of the nick (*Id.*). Accordingly,

the results achieved with the present invention are unexpected and clearly demonstrate the patentability of claim 5 as amended (*Id.*).

It is well settled that evidence of unexpected results will rebut a *prima facie* case of obviousness. *In re Fenn*, 639 F.2d 262, 208 USPQ 470 (CCPA 1981); *In re Murch*, 464 F.2d 1051, 175 USPQ 89 (CCPA 1972). Although it is applicants' position that there is no *prima facie* case of obviousness, even if there were, it would be rebutted by the above experimental data. More particularly, there is an expectation of greater fidelity with the discriminating nucleotide at the 5' end of the ligation junction. Applicants' experimental results, demonstrating the usefulness of the claimed process even at low target DNA quantities and greater fidelity with the discriminating nucleotide at the 3' end of the ligation junction, is highly unexpected. Accordingly, this experimental data obviates any rejection based on the combination of Wu, Takahashi, and Landegren.

Therefore, the rejection under 35 U.S.C. § 103(a) over Landegren, Wu, and Takahashi, is improper and should be withdrawn.

In view of all of the foregoing, applicants submit that this case is in condition for allowance and such allowance is earnestly solicited.

Respectfully submitted,

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